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14. ABSTRACT

We hypothesize that targeted molecular intervention can preserve vision threatened by battlefield trauma-induced corneal andretinal inflammation, corneal and retina/optic nerve apoptosis, ocular surface dry eye after refractive surgery, and retinaldegeneration. We are studying the consequences of trauma-induced (1) corneal inflammation using a gene therapy approach ofproviding soluble Fas ligand to the cornea to determine if this ligand can suppress corneal inflammation in mice; (2) retinalinflammation by examining if transforming growth factor-beta, thrombospondin, and somatostatin, in subretinal space, cansuppress inflammation within retina secondary to autoimmune uveoretinitis and light-induced damage in mice; (3) corneal celldeath by apoptosis and promote regeneration by identifying the anti-apoptotic gene with the greatest capacity to suppresscorneal cell apoptosis using mice; (4) retinal cell death and regeneration by using mice to determine if systemic treatment withlithium chloride can prevent collateral damage to retinal neurons and promote optic nerve regeneration; (5) dry eye bydetermining how to minimize dry eye after LASIK refractive surgery by developing new tests to predict pre-disposition torefractive surgery induced dry eye; and (6) retinal injury by generating stem cell polymer composites.

15. SUBJECT TERMS
blindness, trauma, eye, cornea, retina, dry eye, refractive surgery, inflammation, optic nerve, regeneration

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DOD FY03 Interim Report Task #5

Introduction

An increasing percentage of battlefield injuries occur to the eye in modern warfare. Even treatable battlefield injuries to the eye can lead to blindness because of collateral damage to adjacent tissues. This blindness results from injury-induced inflammation, cell death, failure to regenerate and repair, and development of scar tissue. Task #5 is one portion of a multidisciplinaray project that addressed corneal blindness resulting from abrasions, burns, and pentrating wounds acting on normal corneas or exaggerated in corneas that have undergone refractive surgery, as well as retinal blindness resulting from physical trauma, infection, or laser-induced injury that destroy retinal nerve cells. In task 5 our goal is to prevent the consequences of trauma to the cornea after refractive surgery by developing strategies to diagnose dry eye syndromes. Our specific objective was to determine if there are individuals in which the goblet cells of the conjunctiva do not respond normally to neural and growth factor stimulation and if this abnormal response predisposes these individuals to developing chronic dry eye after laser refractive surgery. Our three subtasks were to 1: determine if the response of conjunctival goblet cells to nerves and growth factors is reduced in a mouse model of dry eye and if loss of corneal nerves (induced by a corneal wound) alters this response. The loss of corneal nerves by a corneal wound mimics the loss of nerves induced in laser refractive surgery. 2: determine if human goblet cells from normal human controls respond to the growth factor EGF, the β-adrenergic agonist isoproterenol, and the cholinergic agonist carbachol. 3: Determine if patients with reduced goblet cell response will have an increased rate of dry eye symptoms and traumatic complications after laser refractive surgery.

Body

I. Research accomplishments for subtask 1: We have continued our studies on measuring alterations in phosphoprotein levels in mouse conjunctiva following stimulation with EGF, carbachol and isoproterenol. We first continued our study on normal mice. We performed corneal wounding (superficial keratectomy) in 12 week-old Balb-c mice in order to mimic laser refractive surgery.

Corneal wounding was done in the right eye and left eye was kept unwounded. Mice not wounded in either eye were considered as the controls. The conjunctiva was isolated from both eyes at different time points (2 days, 6 days, 2 weeks and 4 weeks) following wounding and divided into four pieces. Long time points were used as our goal is to study chronic dry eye that develops after surgery in a percentage of individuals rather than the acute dry eye that occurs in almost everyone after this surgery. Conjunctival tissue pieces were stimulated with no additions (basal), EGF (10^{-7} M,) carbachol (10^{5} M), and isoproterenol (10^{-5} M) for 5 minutes at 37° C in a water bath. The reaction was stopped in keratinocyte basal medium kept at 4° C. The conjunctival lysates were prepared by homogenizing the tissue in RIPA buffer. BioRad multiplex assay was done to study the levels of various phosphoproteins in the conjunctival lysate samples and to determine alterations in the phosphoprotein levels following corneal wounding. Seven-plex assay kit was used to measure the levels of phosphorylated ERK (p42/p44 mitogen-activated protein kinase), JNK, P38 mitogen activated protein kinase, AKT, IκB alpha, STAT-3 and P70S6 at the same time in each sample. Phosphoproteins levels were standardizes to the amount of total ERK (phosphorylated and non-phosphorylated) in each sample. The data were analyzed using Bioplex manager software and fold increase in phophoprotein levels were determined over the basal levels. Statistical analysis was done using student T test and $P < 0.05$ was considered as significant.

A. Results from non-wounded control mice:

Table 1 summarizes the results from 12 week-old control (n=7) mice. We found:

1. Significant increase in phosphorylated ERK following stimulation with EGF and carbachol.
2. Significant increase in phosphorylated AKT following stimulation with EGF and carbachol.
3. Significant increase in STAT-3 following stimulation with isoproterenol.
4. Decrease in the levels of P70S6 were seen following stimulation with isoproterenol, although it was not statistically significant.

B. Results from the wounded (right) eye 2 days following corneal wounding :

Table 2 summarizes the results in the wounded eye 2 days after superficial keratectomy performed in 12 weeks old Balb-c mice (n=5). We found:

1. Significant increase in phosphorylated ERK following stimulation with EGF and carbachol.
2. Significant increase in phosphorylated JNK following stimulation with EGF and carbachol.
3. Significant increase in phosphorylated P38 MAPK following stimulation with EGF.

C. Results from the unwounded (left) eye 2 days following corneal wounding :

Table 3 summarizes the results in the unwounded eye 2 days after superficial keratectomy performed in 12 weeks old Balb-c mice (n=5). We found:

1. Significant increase in levels of phosphorylated ERK following stimulation with EGF alone.
2. Significant increase in phosphorylated JNK following stimulation with carbachol alone.
3. Significant increase in phosphorylated P38 MAPK following stimulation with EGF and carbachol.
4. Significant increase in phosphorylated AKT following stimulation with isoproterenol.
5. Signicant increase in phophorylated IκB alpha, STAT-3 and P70S6 following stimulation with carbachol.

D, Summary of experiments done to date

Table 4 summarizes the number of experiments done to date.

We have done:

1. Seven stimulation experiments on unwounded mice and analyzed all seven with multiplex.
2. Five stimulation experiments on mice 2 days after wounding and analyzed all five with multiplex.
3. Four stimulation experiments on mice 6 days after wounding and analyzed three with multiplex.

4. Six stimulation experiments on mice 2 weeks after wounding and analyzed four with multiplex.
5. Six stimulation experiments on mice 4 weeks after wounding and analyzed three with multiplex.

E. Future experiments for subtask 1

For subtask 1 we will finish the multiplex analysis for the normal mice. We will then perform similar experiments on a mouse model of dry eye the MRL/MpJ-*Fas*^{lpr}/J mice. Mice will be used at 12 weeks of age as the disease has developed by this time. We will compare the results on the diseased mice with those that we have already obtained on the non-diseased Balb/C mice.

Table 1. Alterations in phosphoprotein levels in non-wounded Balb-c control mouse conjunctiva following stimulation with EGF, Carbachol and Isoproterenol using BioRad multiplex assay

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
Phospho ERK	1.35	0.0018 *	1.34	0.0042 *	1.02	0.87
Phospho JNK	1.34	0.053	1.29	0.076	1.14	0.26
Phospho P38	1.06	0.609	0.96	0.73	1.07	0.39
Phospho AKT	1.49	0.044 *	1.22	0.002 *	1.05	0.79
Phospho IkB-alpha	1.41	0.196	1.22	0.07	0.98	0.79
Phospho STAT-3	1.27	0.09	1.32	0.09	1.19	0.012 *
Phospho P70-S6	1.24	0.43	1.30	0.38	0.88	0.18

* $P < 0.05$
N= 7 experiments
Age of mice: 12 weeks old Balb-c mice

Table 2. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (Rt) eye following stimulation with EGF, Carbachol and Isoproterenol 2 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.36	0.0006 *	1.14	0.016 *	0.88	0.07
Phospho JNK	1.44	0.0006 *	1.29	0.0004 *	1.23	0.11
Phospho P38	1.23	0.019 *	1.19	0.22	1.12	0.41
Phospho AKT	1.63	0.39	1.27	0.13	0.87	0.16
Phospho IkB-alpha	1.04	0.31	1.12	0.07	1.03	0.72
Phospho STAT-3	1.23	0.29	1.15	0.21	1.26	0.23
Phospho P70-S6	2.15	0.21	1.53	0.24	1.34	0.39

* P <0.05
N= 5 experiments
Age of mice: 12 weeks old Balb-c mice

Table 3. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 2 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.59	0.0001 *	1.23	0.06	1.08	0.46
Phospho JNK	1.18	0.17	1.54	0.02*	1.12	0.16
Phospho P38	1.29	0.04 *	1.28	0.007 *	1.12	0.14
Phospho AKT	1.35	0.21	1.52	0.50	1.66	0.03*
Phospho IkB-alpha	1.17	0.11	1.13	0.01 *	1.08	0.19
Phospho STAT-3	1.13	0.32	1.33	0.04 *	0.92	0.49
Phospho P70-S6	1.24	0.19	1.49	0.009 *	0.89	0.47

* P <0.05
N= 5 experiments
Age of mice: 12 weeks old Balb-c mice

Table 4. Mouse conjunctival stimulation experiments for multiplex analysis of phosphoproteins

Experiment type	No. of experiments done	No. of experiments analyzed by multiplex	No. of experiments to be analyzed by multiplex
Unwounded	7	7	0
2 day post-wound	5	5	0
6 day post-wound	4	3	1
2 week post-wound	6	4	2
4 week post-wound	6	3	3

II. Research Accomplishments for Subtask 2: As Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary, we revised our IRB protocol to remove him as the doctor to whom adverse advent would be reported. We will begun on this subtask as soon as our project is approved. This subtask must be completed before Subtask 3 can be begun.

III. Research Accomplishments for Subtask 3: As Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary, we were able to enlist COL Kraig S. Bower and LTC Charles Coe from Walter Reed Army Medical Center as new collaborators. For this subtask we have written the IRB protocol for this substask. It was recently approved by their IRB board. The approved protocal and consent form were sent to the IRB at the Schepens Eye Research Institute where is will be reviewed at the next meeting. After approval has been obtained from this IRB it will be sent to the IRB board at the Department of the Army.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a method for measuring multiple second messengers in a single conjunctival sample using bioplex technology.

- Found that Balb/C mice rather than MRL/Mpj mice were the best control of the MRL/MpJ-*Fas*^{lpr}/J mouse model of dry eye.
- EGF and cholinergic agonists cause changes in phosphoproteins ERK and AKT in control, unwounded, mice, but β-adrenergic agonists alter STAT-3 and perhaps P79S6.
- Two days after wounding the wounded and unwounded eyes respond differently from each other and differently from the control unwounded mice.

REPORTABLE OUTCOMES

Konomi K., Azar D., Dartt D.A. Alteration in Signaling Pathways in Mouse Conjunctiva after Corneal Wounding Mimicking Nerve Loss in LASIK Surgery, Invest. Ophthalmol. Vis. Sci. 2006 47: E-Abstract 4600.

CONCLUSIONS

We conclude that in the normal mouse conjunctiva, the epithelial cells differentially respond to growth factors, cholinergic and b-adrenergic agonists. Corneal wounding that mimics laser refractive surgery changes this response.

APPENIDX

1. Konomi K., Azar D., Dartt D.A. Alteration in Signaling Pathways in Mouse Conjunctiva after Corneal Wounding Mimicking Nerve Loss in LASIK Surgery, Invest. Ophthalmol. Vis. Sci. 2006 47: E-Abstract 4600.

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Alteration in Signaling Pathways in Mouse Conjunctiva after Corneal Wounding Mimicking Nerve Loss in LASIK Surgery

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Abstract

Purpose: To determine if the response of conjunctival cells to nerves and growth factors is altered in a mouse model of dry eye and if loss of corneal nerves (induced by a corneal wound mimicking refractive surgery) alters this response.

Methods: Four week old, female BALB/c mice and 12 week old, female MRL/MPJ Faslpr mice were used as nonö dry eye and dry eye models.(n= 9, each strain) Corneal wounds were made through the stromal layer in 6 of 9 mice in each strain by using a trephine, mimicking nerve loss in LASIK surgery. Conjunctival tissue was collected from unwounded eyes and from eyes 2 and 6 days after wounding. Tissue was incubated for 5 minutes with keratinocyte basal media alone, the cholinergic agonist carbachol (Cch) ($10^{ö5}$M), EGF($10^{ö7}$M), and the betaöadrenergic agonist Isoproterenol ($10^{ö5}$M). The amount of the phospho (activated) proteins AKT (known as protein kinase B), extracellular signalöregulated kinase (ERK), cöJun Nöterminal kinase (JNK) and p38 mitogenöactivated protein kinase (MAPK) were measured by multiöplex assay. Results were standardized by measuring the amount of total ERK.

Results: Under basal conditions, the amount of phospho JNK in unwounded eyes and phospho ERK 6 days after wounding was higher in the dry eye model than in the nonödry eye model (p= 0.046, 0.033, respectively). Compared to basal, Cch stimulation of JNK phosphorylation in unwounded eyes was lower in the dry eye model than in the nonödry eye model (p=0.048). In the nonödry eye model Cch increased the

phosphorylation of p38 MAPK above basal in unwounded eyes (p=0.02), but this stimulation was decreased in tissue 6 days after wounding (p=0.004). Cch did not significantly alter AKT activation. EGF and isoproterenol did not significantly alter activation of any phosphoproteins measured.

Conclusions: In a mouse model the response of conjunctival cells to cholinergic agonists appears to be altered by dry eye status and loss of corneal nerves (as could occur in refractive surgery).

Key Words: conjunctiva ð phosphorylation ð cornea: tears/tear film/dry eye

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